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EVALUATION OF AN IMMOBILIZED CELL BIOREACTOR FOR DEGRADATION OF META- AND PARA-NITROBENZOATE

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PREFACE

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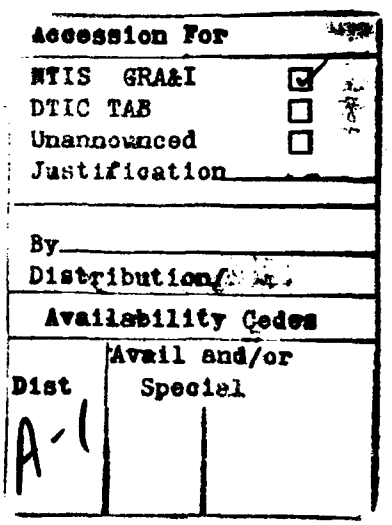

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EVALUATION OF AN IMMOBILIZED CELL BIOREACTOR FOR DEGRADATION OF META- AND PARA-NITROBENZOATE

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Abstract

Meta- and para-nitrobenzoic acid (m-NBA, p-NBA) are pollutants found in waste streams from metal-stripping processes utilizing cyanide-free solvents. The Kelly AFB Industrial Waste Treatment Plant (IWTP) is currently incapable of removing these compounds from the waste water it receives because of (1) the presence of significant quantities of ethylenediamine, a preferred substrate, and (2) an upper limit of 4.5 hours on the hydraulic residence time in the IWTP. This work describes the enrichment and preliminary characterization of a microbial consortium capable of utilizing both m-NBA and p-NBA as sole carbon sources. Experimental results indicate that m-NBA degradation involves an oxidation pathway, while p-NBA degradative proceeds through a reductive pathway. This consortium was immobilized by entrapment in alginate beads and grown in a continuous-flow airlift reactor. Single substrate and mixed substrates were fed to the reactor. Conditions were varied to simulate different waste treatment scenarios: switching from one stripping solvent batch to another, starting up of the metal stripping process, mixed solvent batches, and changing the loading rate of substrate to the bioreactor. Results indicate that the nitrobenzoate fraction of the metal stripping waste can be effectively treated in a continuous-flow, immobilized-cell bioreactor with a hydraulic residence time well below 3 hours. Furthermore, the process can be operated over long periods (>250 hours) with little diminution of performance and responds rapidly to changes in substrate.

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INTRODUCTION

Biotreatment

Proliferation of toxic organic compounds that are resistant to photochemical or biological degradation (xenobiotic) is a burgeoning threat to human health. Complications in the treatment of xenobiotics arise due to their chemical composition and the difficulty of their isolation from other compounds. Process waste streams, for example, can be isolated and their chemical composition well defined. On site treatment of these wastes is constrained by the chemical properties of the compounds present (mixed organics, extreme pH, high salinity) in the process streams. Recently, a large body of literature has accumulated regarding the use of microorganisms to degrade a wide variety of halogenated, polycyclic, and multi-substituted organic molecules heretofore thought not to be susceptible to biodegradation[1-4]. While in a few cases, a single organism has been found which can mineralize a specific xenobiotic, most often a consortium of species is necessary to completely detoxify mixtures [5,6]. Exploiting the capability of these organisms is problematic, and care must be taken to establish appropriate bioreactor design and operation. Of primary importance for these efforts will be the long-term maintenance of a population which preserves efficient biodegradative activity.

Many considerations affect the design of bioreactors for waste treatment applications. The compounds involved are often present either at low concentrations that do not support rapid growth or at high concentrations that inhibit growth. Substrate inhibition often results in growth lags, the length of which are directly proportional to the concentration of the inhibitory compound. The composition of the waste mixture may vary over time, as might the loading rates and the volumetric flow of the waste stream. Multiple organisms are usually required to effect the bioremediation of a stream containing several organic compounds.

These considerations mitigate against *the use of suspended cell reactors.* Cell suspensions can be grown to a limited cell density, restricting the degradative activity per unit volume. This implies that for either batch or continuous operation, the reactor must be sized such that the residence time of the fluid in the reactor is sufficient to allow degradation of the compounds. The limitation of cell growth by

high and low concentrations of the compounds will tend to cause suspended cell reactors to be rather large. Continuous flow reactors would enable a higher throughput of material than batch reactors, but the flow rates that can be accommodated are limited by the growth kinetics of the organisms. Mixed cultures can be maintained in batch reactors, but due to differences in growth rates, yields and dependence on environmental conditions, it is extremely difficult to maintain two populations in a continuous flow suspension culture, and virtually impossible to maintain three or more.

The use of immobilized cell reactors can alleviate each of the problems presented above. Immobilized cell reactors have significantly higher cell densities than suspended cell reactors, and the volumetric productivity reflects the ten- to one hundred-fold increase in cell density that can be achieved. Consequently, smaller reactors can be used. Since cell densities are higher and the cells remain in the reactor, degradation rate and cell growth rate are to a large degree uncoupled. This lessens the negative effects of the extremes of substrate concentration. Low concentrations can be fed rapidly since the cell population does not need to grow sufficiently quickly to avoid being washed out of the reactor. Substrate inhibition is also relieved. Immobilizing cells introduces mass transfer resistance due either to the immobilization matrix or the cell layers. This resistance lowers the effective substrate concentration for a fraction of the cells in the reactor, enabling them to actively degrade the compounds without a lag time.

Problem Summary

For many years the United States Armed Forces has utilized cyanide-containing stripping compounds in their metal-refinishing processes. The U.S. Air Force has undertaken a program to completely remove cyanide from these processes, and the effort has reached the demonstration stage in the plating shop of Kelly AFB. The successful demonstration of efficacy of the cyanide-free metal stripping compound CLEPO 204 led to questions surrounding treatment of the stripping wastes in the Kelly AFB Industrial Waste Treatment Plant (IWTP). The composition of spent CLEPO 204 is 33% ethylenediamine, 10% sodium nitrobenzoate and an "unidentified" red compound. Experiments were performed under the direction of the Idaho National Engineering Laboratory to determine the biodegradability of ethylenediamine(EDA) and nitrobenzoate (NBA) using sludge from the Kelly AFB IWTP.

Shake flask tests and continuous flow, bench-scale bioreactor tests were conducted using EDA or spent CLEPO 204 as the substrate. It was found that the shake flask cultures completely degraded EDA when it was the sole substrate. However, using spent CLEPO 204 as the substrate caused a reduction in EDA degradation and less than 20% degradation of NBA within 48 hours.

Continuous-flow tests with a hydraulic residence time of 5.3 hours (similar to that of the IWTP) gave only 8.8% degradation of ethylenediamine when it was the sole carbon source. Increasing the residence time to 8.3 hours led to an 88% removal of EDA but also caused ammonia levels to jump to well over 100 ppm. Spent CLEPO 204 components were not removed in the continuous-flow bioreactor with a residence time of 5.3 hours. Increasing the hydraulic residence time to 8.3 hours led to 100% removal of EDA within 30 hours and NBA within 150 hours. Concomitant with the degradation of these compounds were increases in effluent ammonia and nitrite.

The IWTP is running at or above its designed capacity with a hydraulic retention time of 4.8 hours. The residence time in the plant cannot be further increased. The preliminary conclusion drawn from these results is, therefore, that biological treatment of spent CLEPO 204 using the Kelly AFB Industrial Waste Treatment Plant is not currently feasible.

Research Objective

The objective of this work is to evaluate the feasibility of using a continuous-flow, immobilized cell reactor to remove m-nitrobenzoate and p-nitrobenzoate from aqueous streams. The effects of substrate loading rate, hydraulic residence time, substrate concentration and substrate composition on the fractional removal of nitrobenzoate have been studied. The response to starvation and rapid changes in substrate have also been investigated. These studies indicate the degree of stability of this waste treatment scheme to operational upsets and suggest other variables that might have a significant impact on the performance of this reactor system.

MATERIALS AND METHODS

Isolation of Bacteria

The organisms used in these studies were isolated from an activated sludge sample taken from the Industrial Waste Treatment Plant at Kelly Air Force Base in Austin, Texas. Inocula were diluted 1:10 v/v in Spain's minimal salts medium (SMSB)[7] supplemented with 100 ppm m-nitrobenzoate and grown in 250 mL flasks. The

cultures were diluted 1:10 following an observable change in turbidity, which took 1-2 days following inoculation. A consortium containing at least two distinguishable species resulted from serial cultivation on m-NBA, and these species were designated Kelly 4 and Kelly 7. Kelly 4 grows rapidly on m-NBA concentrations as high as 200 ppm, following a lag period of approximately 18 hours. Nitrite is released. Kelly 4 was not observed to grow on p-NBA. Kelly 7 grows extremely slowly on p-NBA, with concomitant release of ammonia. Strains were maintained on plates containing MSB[8], 18 g/L Bitek agar and 100 ppm m-nitrobenzoate.

Bacterial Immobilization

A suspended cell culture of the consortium was grown in SMSB containing 100 ppm m-NBA at 30° C. A previously autoclaved solution of 4% Na-alginate (Kelco, Manugel "GHB") dissolved in 200mM NaCl was mixed with log phase cells to give a final concentration of 3% alginate. Immobilized cell beads were formed by passing this solution through an 18-gauge needle, allowing individual droplets to fall into the airlift reactor vessel which contained 400 mL of 100mM SrCl at room temperature. Strontium was used as the cross-linking agent because it yields stronger gel beads than those formed using calcium[9]. The airlift and SrCl solution had been autoclaved and sealed so that the process was carried out under sterile conditions. The beads were allowed to cure for 4 hours with no agitation or air sparging. After curing, the reactors were flushed with SMSB to remove excess SrCl. The beads were fluidized by air sparging, and the culture allowed to grow in batch overnight in SMSB containing 100 ppm m-NBA.

Cell Culture

Suspended cell cultures, both batch and continuous, were analyzed for the more rapidly growing of the two microorganisms isolated, Kelly 4. Erlenmeyer flasks (250 mL) were used in studies to determine the maximal growth rate of Kelly 4 at 30° C in SMSB containing 100 ppm m-NBA. The concentration of m-NBA was also monitored to quantify its rate of degradation. Chemostat cultivation of Kelly 4 was also undertaken as a means to determine the dilution rate at which suspended cells would be washed out of a well-mixed reactor. SMSB amended with 100 ppm m-NBA was fed to a New Brunswick Bioflo reactor with a working volume of 500 mL. Temperature was maintained at 30° C, pH at 7.0.

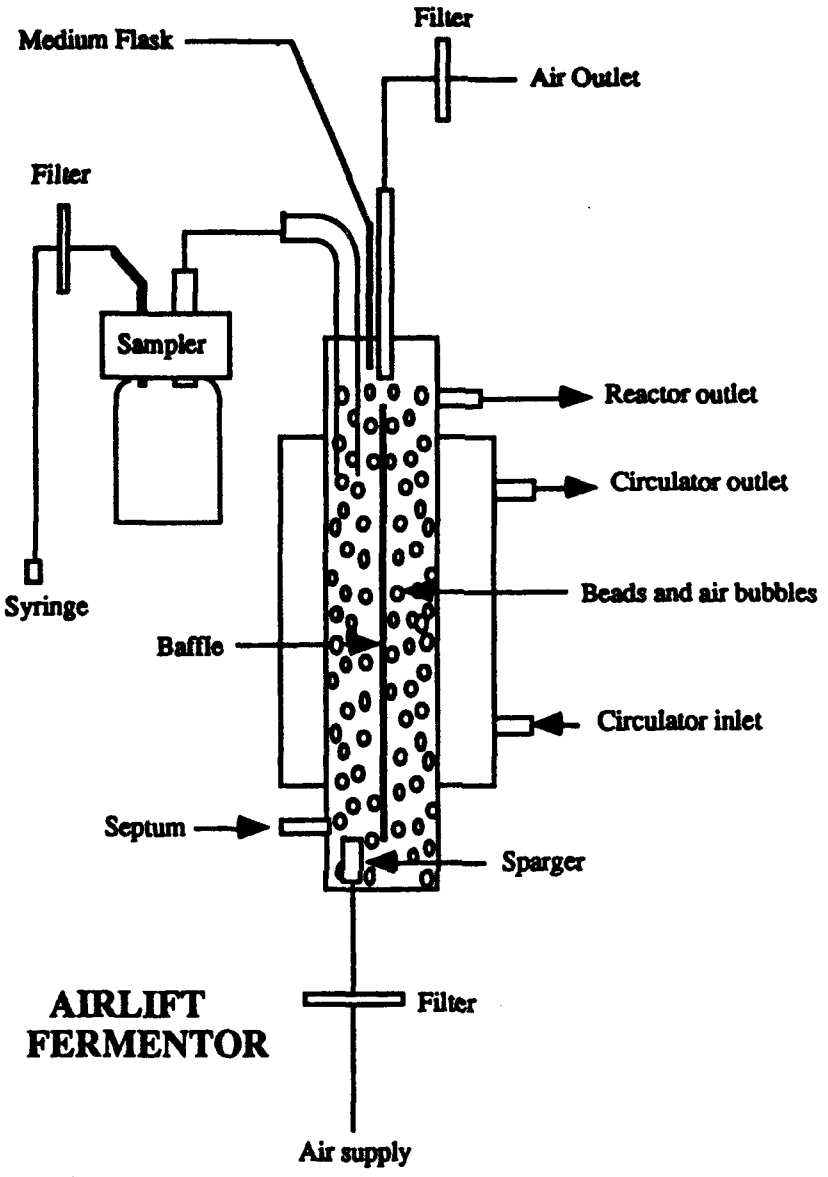

FIGURE 1

Immobilized consortium fermentations were conducted at 30°C in a pair of Kontes airlift vessels with a single central vertical baffle to promote mixing. Figure 1 is a schematic of the reactor used and its operation. Airflow was set so that the beads in the reactor (bead volume between 150 and 200 mL) were well mixed and the baffle remained free of clogging. The working volume in each reactor was maintained at 600 mL by fluid overflow. The original gel bead volume in reactor 1 was 172 mL and in reactor 2 was 185 mL. Final bead volumes were 106 mL and 115 mL, respectively. Medium and bead samples were periodically withdrawn aseptically.

The cell concentration in the medium was determined by measuring absorbance at 600 nm and using a correlation. Both reactors were fed SMSB-based medium with the pH adjusted to 7.5. After batch cultivation to establish culture growth, each reactor was subjected to a different substrate and dilution rate profile. The reactor fluid volumes used to determine dilution rates are those of the fluid volume in the reactor at the time. All dilution rates reported have the units of reciprocal hours (hr^{-1}).

Reactor 1 was subjected to the following sequence: continuous feed of 100 ppm m-NBA at dilution rates of 0.4 and 0.6; a step change to continuous feed of 50 ppm m-NBA at a dilution rate of 1.2; batch cultivation to complete depletion of m-NBA; a step change to continuous feed of 100 ppm m-NBA at dilution rates of 0.67 and 0.33; a step change to continuous feed of 48 ppm p-NBA at dilution rates of 0.33 and 0.1; and a step change to 50 ppm m-NBA at a dilution rate of 0.31 hr^{-1}.

Reactor 2 was subjected to the following sequence: continuous feed of 100 ppm m-NBA at dilution rates of 0.7 , 1.4, and 0.34; a step change to continuous feed of 50 ppm m-NBA at a dilution rate of 0.74 and 0.34; a step change to continuous feed of medium containing 40 ppm of both m-NBA and p-NBA at a dilution rate of 0.32; and a step change to continuous feed of medium containing 80 ppm of both m-NBA and p-NBA at a dilution rate of 0.31 hr^{-1}.

Analytical Methods

When a single substrate was fed to the reactor, the concentrations of m-NBA and p-NBA in the reactor samples were determined using the A_{266} for m-NBA and A_{275} for p-NBA. Calibration curves for both compounds were established in SMSB at pH 7.0. For those reactor runs containing mixed substrate feed, high-pressure liquid chromatography (HPLC) was performed using a mBondapak C_8 column (3.9 mm by 30 cm; Waters Associates, Inc., Milford MA). A linear gradient was run using methanol-water (acidified with trifluoroacetic acid) as the mobile phase. The MeOH:water composition was 50:50 at the start, changing linearly to 40:60 after 3 minutes, then changing immediately to 30:70 for the next 5 minutes. This gave a peak separation time of 40 seconds and no overlap of peaks. Compounds were detected by their absorbance at 270 nm with a Hewlett Packard diode array detector. Concentrations were quantified using peak areas and calibration curves established using pure components and mixtures of m-NBA and p-NBA.

Alginate bead samples were removed from the reactor at specified times and stored at 4° C for no more than a week prior to processing. The volume of the

sample was determined by decanting the residual medium, and estimating the packed bead volume using a graduated conical centrifuge tube. Approximately 1 mL of beads from each sample were dissolved with buffered saline solution[9]. The cells were precipitated by centrifugation in a Sorvall SS34 rotor at 8,000 rpm for 15 minutes. The cells were resuspended in 990 mL of 20 mM sodium phosphate pH 7.0, and lysed by adding 10 mL of 5 M NaOH and placing the samples at 100 °C for 15 minutes. A Pierce BCA total protein determination kit was used to analyze the samples. Total bead volume collected was noted throughout the course of the experiments, so that an estimate of total protein present in the reactor at any given time could be made.

RESULTS

Cell Growth

Figure 2 indicates that the maximal growth rate of Kelly 4 on 100 ppm m-nitrobenzoate in SMSB is approximately 0.36 hr^{-1}. The degradation rate of the m-NBA under these conditions is 186 mg hr^{-1} L^{-1} OD_{600}^{-1}. This implies that the washout dilution rate in a chemostat should be roughly 0.36 hr^{-1}. Figure 3 shows the results of a chemostat fermentation of Kelly 4 at 30°C, pH 7.0. The culture is washed out at a dilution rate close to 0.2 hr^{-1} rather than the expected 0.36 hr^{-1}. The discrepancy between the performance *in batch and continuous suspension* culture is surprising. One possible explanation is substrate inhibition by m-NBA.

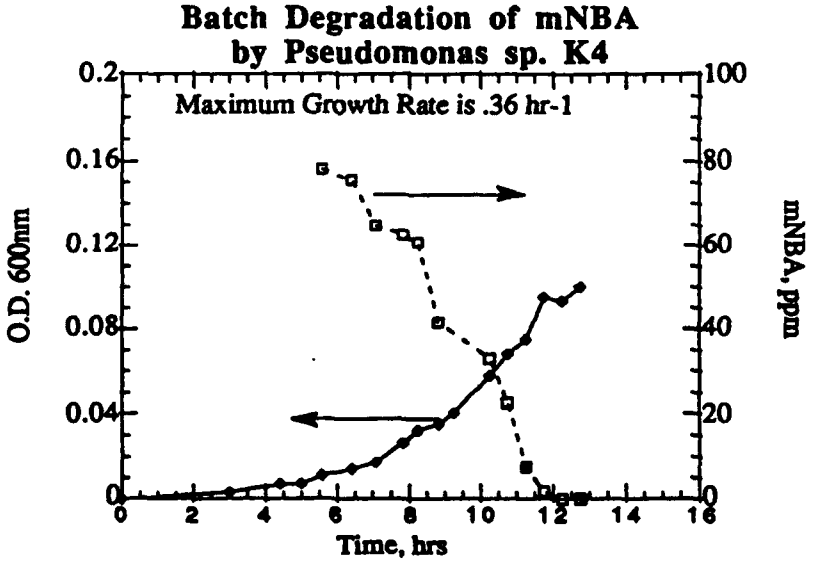

FIGURE 2

The maximal growth rate in shake culture was achieved at m-NBA concentrations in the range of 40 to 65 ppm. This hypothesis is supported by the observation that cells actively growing in shake culture exhibit some lag in growth upon sub-culturing into 100 ppm m-NBA.

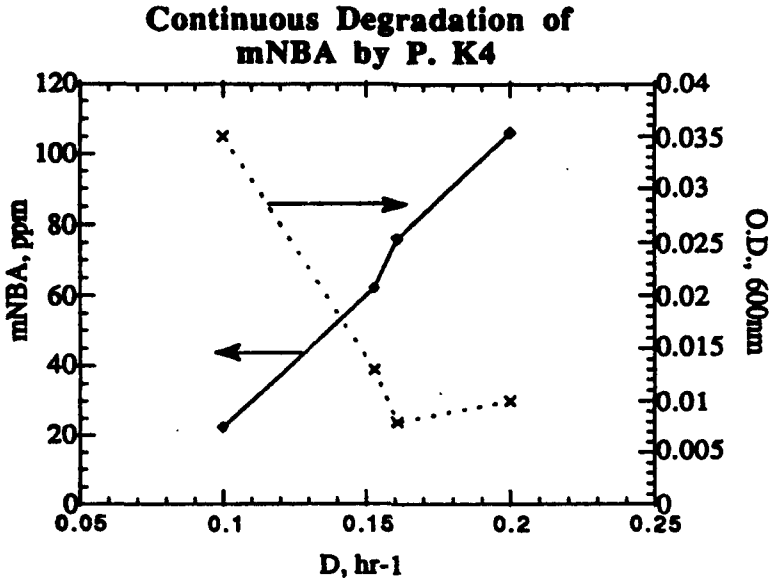

FIGURE 3

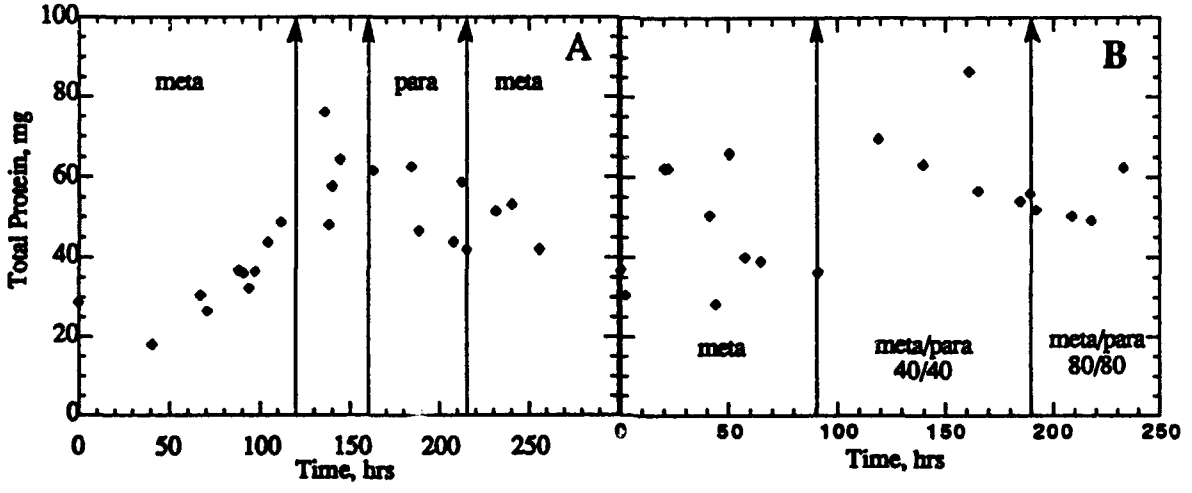

FIGURE 4

Total Protein

Figures 4A and B indicate the total protein in reactors 1 and 2, respectively, as a function of time. The results for reactor 1 indicate that it took nearly 150 hours to fully load the beads with biomass. This is supported by the observation that before

about 120 hours, the A_{600} of the fermentation broth was essentially zero, indicating no cells released from the gel beads. The gradual decline in total protein in reactor 1 after 150 hours can be accounted for by the volume of beads removed during sampling. Initially, the biomass in reactor 2 grew more rapidly, probably because it was the first reactor in which beads were formed and to which growth medium was introduced. As with reactor 1, the subsequent decline in total protein in the reactor can be attributed to gel bead removal.

Reactor 1 Performance

Starvation

Figure 5 illustrates the degradative performance of the consortium in reactor 1 following a period of starvation. The solid line and diamonds represent the loading rate of m-NBA into reactor 1 as a function of time. A 15 hour period of starvation is followed by rapid pumping of m-NBA into the reactor. The outlet concentration of m-NBA increases transiently, followed by complete degradation of the compound. The transient increase in m-NBA in the reactor is due solely to mixing. There is no indication of a loss of degradative activity or of a lag phase following this period of starvation. The pH in the reactor remained roughly constant during the starvation and post-starvation periods at 6.9

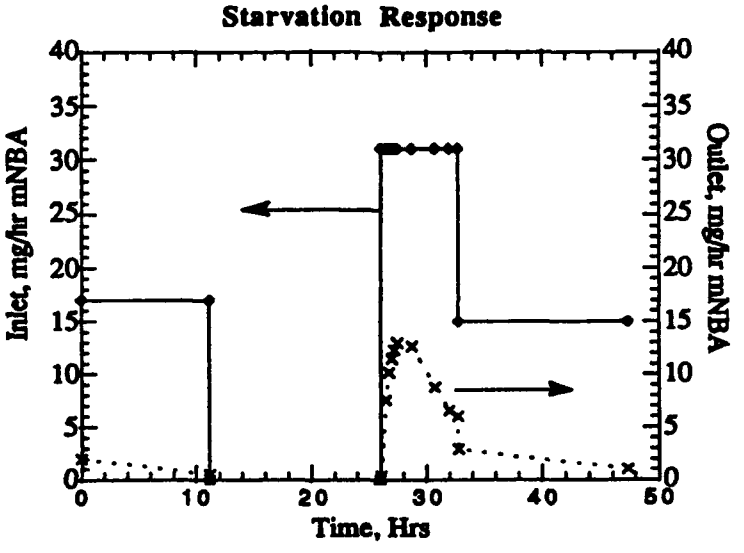

FIGURE 5

Shift from m-NBA to p-NBA

The inlet and outlet concentrations of p-NBA following a shift from m-NBA feed to p-NBA feed are shown in Figure 6. This culture was previously exposed to only m-NBA as a carbon source. The outlet concentration of p-NBA following the switch from m-NBA indicates the immediate degradation of some of the p-NBA. Subsequently, this reactor degraded about 60% of the p-NBA fed. The pH in the reactor rose to 7.5, probably due to the accumulation of ammonia released during the metabolism of p-NBA.

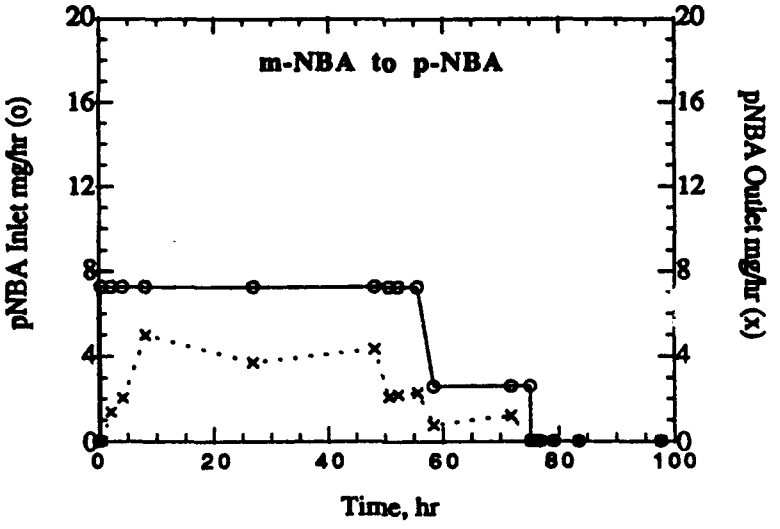

FIGURE 6

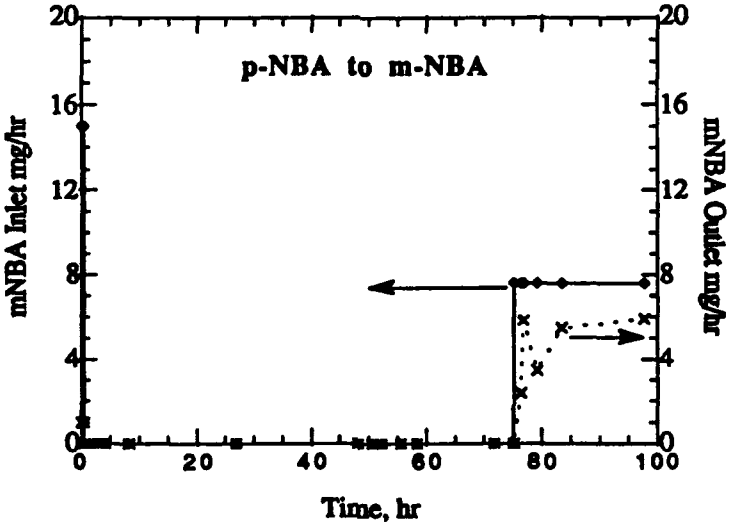

FIGURE 7

Shift from p-NBA to m-NBA

Figure 7 shows the concentration of m-NBA in the inlet and outlet streams following a switch in feed from p-NBA to m-NBA. The m-NBA feed had a pH of 7.1 instead of 7.5 due to the elevation of reactor pH caused by p-NBA degradation. Only approximately 20% of the influent m-NBA was degraded, compared to the greater than 90% degradation exhibited 50 to 100 hours earlier. The apparent inability of the reactor to exhibit the degradative performance it displayed at an earlier time may be due to the ammonia produced during the metabolism of p-NBA. The pH of reactor was 7.5 immediately prior to the switch in substrate and took over 20 hours to drop to 6.8.

Reactor 2 Performance

Degradation of m-NBA

The carbon and energy sources provided to reactor 2 were m-NBA and mixtures of m-NBA and p-NBA. It is evident from the outlet concentrations for m-NBA displayed in Figure 8A for the straight m-NBA feed that the consortium is capable of degrading nearly 100% of the m-NBA at contact times below 3 hours. The addition of a moderate level of p-NBA (40 ppm) to the feed has essentially no effect on the degree of degradation of m-NBA. However, increasing both the m-NBA and p-NBA concentrations to 80 ppm in the feed results in a loss of the majority of the degradative activity. This is indicated by the rapid and sustained elevation in outlet m-NBA concentration following the increase in feed concentration.

Degradation of p-NBA

Figure 8B displays the inlet and outlet concentrations of p-NBA for reactor 2 during the introduction of mixtures of p-NBA and m-NBA to the vessel. Despite being fed only m-NBA for over 100 hours at the time of introduction of p-NBA to the reactor, the p-NBA was rapidly and completely degraded following a short mixing period. This performance continued unabated in the face of a concomitant doubling of m-NBA and p-NBA concentrations in the feed.

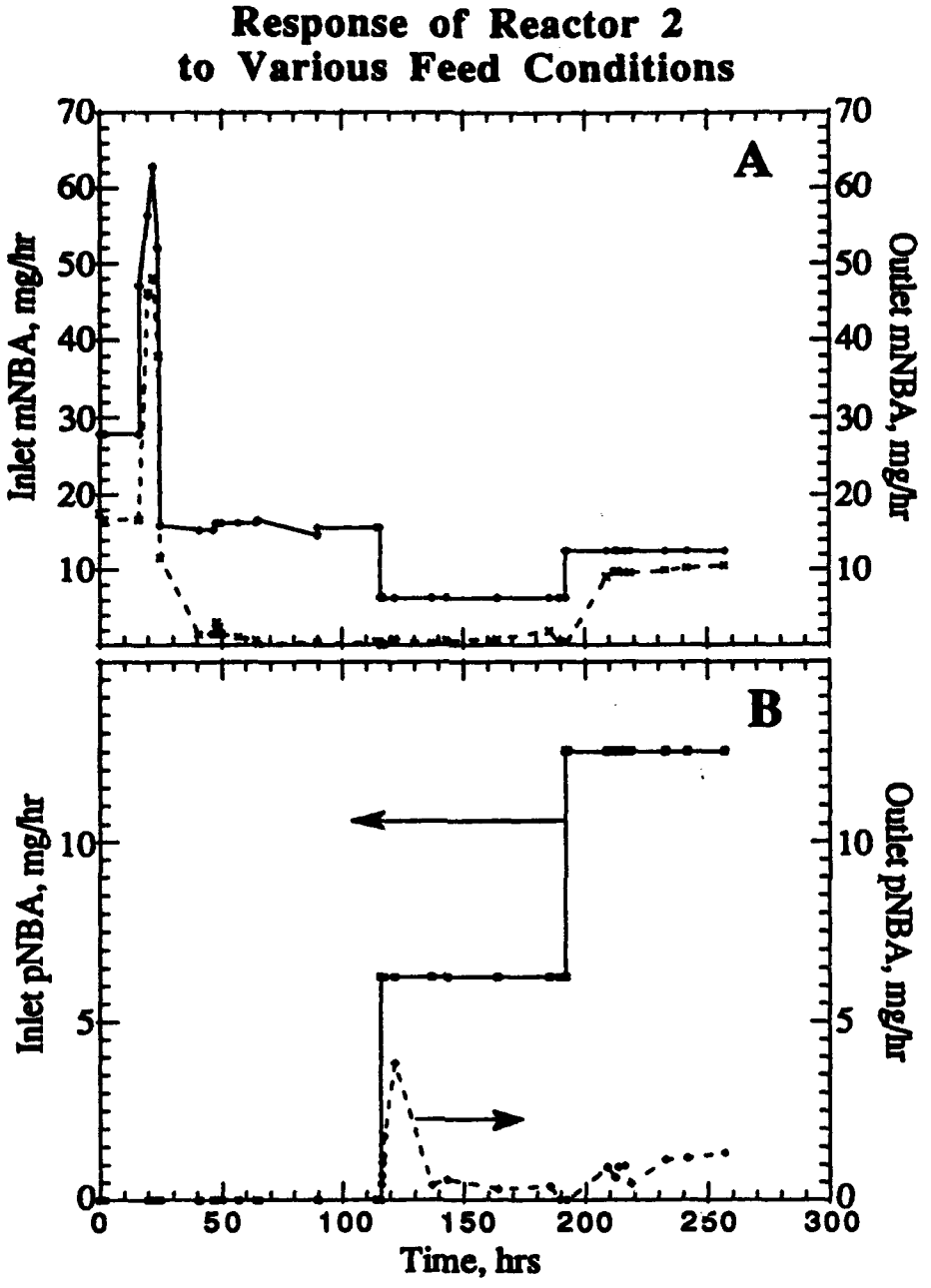

FIGURE 8

DISCUSSION

These studies indicate that the combination of organisms evaluated and the immobilized cell bioreactor represents a feasible option for the treatment of aqueous

streams containing mixtures of m- and p-nitrobenzoate. The system exhibits the capability to degrade both pure substrate and mixed substrate feeds. The system responds rapidly to shifts in substrate, exhibits stable activity over time, is insensitive to moderate starvation times (15 hours), and is effective over a wide range of loading rates.

Comparing the degree of p-NBA degradation in reactor 1 under single substrate conditions with that for reactor 2 under mixed substrate conditions suggests that p-NBA metabolism is enhanced by concurrent metabolism of m-NBA. Only about 60% of the p-NBA was degraded in reactor 1 in the absence of m-NBA, versus greater than 95% degradation in reactor 2 when both m-NBA and p-NBA were fed at a concentration of 40 ppm. Conversely, the metabolism of m-NBA appears relatively unaffected by p-NBA degradation at lower p-NBA concentrations (40 ppm) but negatively affected at higher levels (80 ppm).

It is hypothesized that each isomer is metabolized by a different organism, though Kelly 7 is expected to degrade both isomers. The interaction between Kelly 4 and 7 is probably mediated by an extra-cellular product. A plausible hypothesis for the beneficial affect of m-NBA metabolism on p-NBA metabolism involves the pH inside the gel beads. We have established that p-NBA metabolism is reductive, releasing ammonia, while m-NBA metabolism is oxidative, releasing nitrite. The medium was initially at a pH of 7.5. In the absence of nitrite generation, the release of ammonia would cause the pH within the beads to rise significantly during p-NBA metabolism, inhibiting further cell growth or p-NBA metabolism. The rate of p-NBA metabolism would then be limited by the diffusion of ammonia out of the gel beads. If nitrite were being generated as well as ammonia, the pH change would depend upon the relative rates of generation of nitrite and ammonia. Calculations based on the diffusivity of ammonia in alginate and a bead diameter of 3 mm suggests that the pH inside the bead could rise above 8.0 given the observed rates of p-NBA metabolism.

This mechanism of interaction is consistent with the observation regarding the inhibition of m-NBA metabolism at higher p-NBA levels. In this medium, ammonia is the nitrogen source for the m-NBA degrader. At low relative rates of p-NBA metabolism, the greater availability of a nitrogen source inside the bead would be advantageous to the m-NBA degraders and would alleviate the acidic inhibition of growth that is often caused by nitrite generation. As the rate of generation of ammonia increases, the pH in the beads would rise, possibly to an inhibitory level.

This would explain the observation that the rate of m-NBA degradation per unit protein in reactor 2 drops 60% when the feed concentration goes from 40:40 to 80:80.

The assertion made regarding substrate inhibition is supported by certain details of Figure 9, which shows the percentage removal of m-nitrobenzoate as a function of reactor loading rate. Squares denote a feed concentration of 50 ppm m-NBA while circles denote 100 ppm. Data are taken from both reactors during the initial periods when only m-NBA was fed. Two trends are indicated. First, percentage removal drops as loading rate increases. Second, lower feed concentration leads to higher percentage removal. Of particular interest are the two data points connected by the arrow. These points were achieved sequentially. In going from point 1 to 2, the concentration of m-NBA in the feed was changed from 100 ppm to 50 ppm and the flow rate was doubled. The jump in percent removal indicates that the degradative activity in the reactor was inhibited by the higher m-NBA concentration.

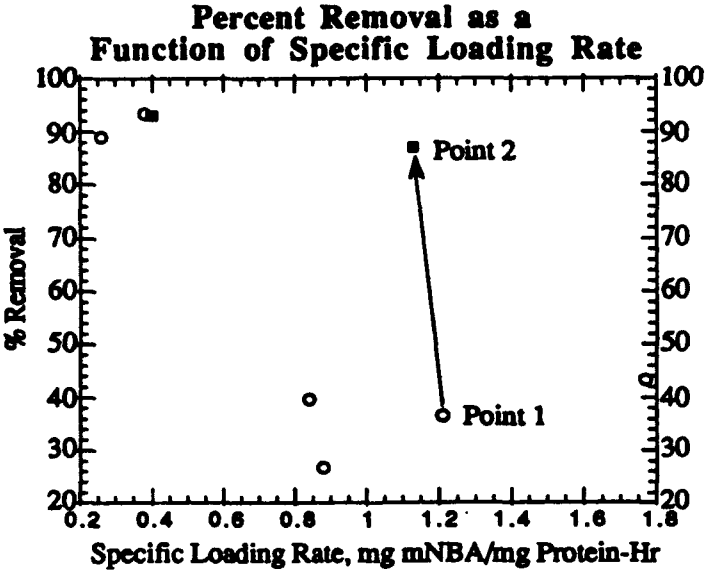

FIGURE 9

Another factor likely to contribute significantly to the behavior exhibited is the composition of the population in the reactor. As shown in Figure 6, the p-NBA concentration in reactor 1 decreases continually over time. The trend might indicate the replacement of m-NBA degraders by p-NBA degraders in the reactor. The transient is of too great a duration to be explained simply by induction of the p-NBA pathway. Population shifts could also explain the changes in degradative activity under mixed substrate feed conditions. The decline in m-NBA degradative activity

following the shift from 40:40 to 80:80 could be caused by a decline in the population most active in m-NBA metabolism.

CONCLUSION

Meta-nitrobenzoate and para-nitrobenzoate can be degraded rapidly and completely using a consortium initially isolated from the IWTP at Kelly AFB. The degradation occurs when either isomer is the sole carbon source or when both are present. Preliminary metabolic characterization of the consortium indicates that m-NBA degradation involves an oxidation pathway, while p-NBA degradation proceeds through a reductive pathway. Under certain cultivation conditions in an immobilized cell reactor, concurrent metabolism of both isomers is synergistic, leading to more rapid and complete degradation of each compound than observed in single-substrate studies. Under other conditions, the interaction appears inhibitory. Clarification of the mechanisms underlying these observations is necessary before reactor performance can be maximized. The pH sensitivity of the organisms involved is probably an important determinant and must be investigated more fully. Combined with measurements of the pH gradient inside the gel beads under different operating conditions, this information would help establish the effect of pH, nitrite and ammonia generation on degradation rates. In addition, enumeration of the population size of each species in the reactor, and elucidation of the spatial distribution of each species in the gel beads, are necessary to more clearly define the local environments these organisms experience. With such information, realistic models of reactor performance can be formulated and used to design and operate waste treatment systems effectively.

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